IMPAIRED NATURAL KILLER CELL ACTIVITY ASSOCIATES WITH INCREASED SUSCEPTIBILITY TO INFLAMMATION INDUCED BY HERPESVIRUS INFECTION IN PATIENTS WITH NICKEL HYPERSENSITIVITY

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Nickel (Ni) is one of the most common causes of allergic contact dermatitis (ACD) and the number one allergen in the frequency of positive patch test reactions. The cellular basis of ACD is the lack of specialized T cells with regulatory function allowing for the expansion of $Ni\frac{I}{N}$ specific cytotoxic CD8+ T cells. However, Ni also exerts a number of not yet fully understood activities on cells belonging to the immune system. To this regard, an immunosuppressive activity of Ni on natural killer (NK) cells has been repeatedly suggested both in animal models and humans. Here we demonstrate that NK activity of $Ni\frac{I}{N}$ intolerant patients was significantly lower than observed in controls. Moreover, the addition of $Ni\frac{I}{N}$ sulphate to cultures of peripheral blood lymphocytes (PBL) obtained by $Ni\frac{I}{N}$ allergic patients, further depressed the NK cell activity. Finally, according to the key role exerted by NK cells in containing viral infections, we found an increased frequency of Herpesvirus 1 (HSV-1) recurrence among patients with $Ni\frac{I}{N}$ allergy. In conclusion, our results indicate that $Ni\frac{I}{N}$ allergic patients may present reduced NK cell response, this resulting in turn in increased susceptibility to viral infections. Patch testing should be considered in individuals affected by unexplained recurrences of HSV-1 infection.

The transition metal Nickel (Ni) is a hapten with a high immunogenic potential. In fact, Ni hypersensitivity represents a very common human disease state, mainly occurring in females, defined as allergic contact dermatitis (ACD). It is now well documented that the maintenance of a non-allergic condition to environmental chemicals depends upon the expansion of a recently described subset of lymphocytes, the regulatory T cells. Therefore, the development of undesired allergic reaction to metals appears as the consequence of a defective function of this regulatory T cell subset (1).

However, evidences exist indicating Ni as an important modulator of immune responses, too. Initial studies on the immunologic effects of Ni have documented an impaired activity of both T and NK cells in mice treated with $Ni\frac{I}{N}$ chloride whereas no alterations in the B cell response to T cell independent antigen or in the phagocytic capacity of macrophages could be demonstrated in Ni-treated mice (2-3). According to these findings, it is demonstrated that Ni enhances mortality in mice infected with a sublethal dose of murine cytomegalovirus (MCMV) and reduces virus-

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augmented NK cell activity (4). More recently, it has been shown in humans that Ni modulates surface receptors expression, reduces phytohemagglutinin (PHA)-driven lymphoproliferation, and upregulates some proinflammatory cytokines production, including interferon (IFN)-gamma (5). Moreover, similarly to that observed in the mouse model, a depressed NK activity has been demonstrated in patients with Ni ACD (6). NK cells are an important cellular feature of innate immunity responding in an antigen-independent manner to help contain viral infections before the development of adaptive immune responses. NK cells are found in the peripheral circulation, in the spleen, and bone marrow and, like other leukocytes, they can be recruited to sites of inflammation by chemokines and other chemoattractants. They appear to be important for the control of tumors *in vivo* and serve a critical function in host defence against viral infections, especially those caused by members of the herpesvirus family (7-8).

Interestingly, differences have been demonstrated between Ni-allergic and non-allergic subjects in T cell activity and production of cytokines when peripheral blood lymphocytes (PBL) are stimulated by $Ni\frac{I}{N}$ sulphate. Consistently with these observations it has been demonstrated that the uptake of $^{63}Ni\frac{I}{N}$ isotope into blood lymphocyte nuclei of Ni-allergic subjects is significantly higher than into those of the control group.

We can speculate that the impairment of both T and NK activities could induce an undefined form of immunodeficiency in Ni-sensitized patients. Since intact immune responses are required to effectively protect the host against the spread of infectious agents, an increased susceptibility to viral infections should be observed in $Ni\frac{I}{N}$ allergic patients. In this report, we confirm and extend our previously reported data on depressed NK activity in patients with Ni allergy (6). In addition, we describe, for the first, time an association between Ni hypersensitivity and increased susceptibility to infection, primarily recurrence of Herpesviruses (HSV) infection.

MATERIALS AND METHODS

Study population

Fifty five patients (fifty one women and four men; mean age 37 years, range 18-63), with confirmed Ni ACD, participated in the study. Twenty five healthy volunteers (twenty women and five men; mean age 36 years; range 26-60) were included as controls. All subjects were patch tested with the European standard series of contact allergen (F.I.R.M.A. Spa, Florence, Italy). A diagnosis of ACD to Ni was based on personal history, clinical examination and a positive patch test to $Ni\frac{I}{N}$ sulphate. Patients with positive patch test reactions to other contact allergens besides Ni were excluded from the study. Subjects with high levels of total serum IgE (Radim SpA, Pomezia, Italy) and positive prick tests (Stallergenes, Saronno, Italy) to most common food and inhalator allergens were also excluded from the study. All the subjects gave their informed written consent according to the Ethical Committee of the University of Rome, La Sapienza, Italy.

Patch testing

All subjects were patch tested 3-6 months before blood samples were collected. The allergens were applied to the back for 48 hours with Finn chambers (F.I.R.M.A. SpA, Florence, Italy). Readings were made at 3 and 6 days after application using the International Contact Dermatitis Research group guidelines (9). The inclusion criterion for patients with ACD was a ++ or +++ reaction.

Isolation of PBL

PBL were isolated from heparinized venous blood by Ficoll-Hypaque density-gradient centrifugation (Lympholyte-H; Cedarlane Laboratories, Hornby, Ontario, Canada) and washed twice in phosphate buffered saline (PBS), pH 7.4. PBL were then cultured in 24-well plates at a density of 1 x 10^6cells/ml in RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Euroclone, Pero, Italy), 2mM glutamine (Sigma, St Louis, MO, USA) and 50 μg/ml gentamycin (Sigma).

Measurement of NK cell activity

NK cell activity was tested as effector cells versus K562 target cells labelled with ^{51}Cr (Nycomed Amersham Sorin srl, Saluggia (VC), Italy). K562 target cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin-streptomycin (100 UI/ml – 100 μg/ml) and 2 mM glutamine, labelled for 1 hour with ^{51}Cr as sodium chromate, at 37°C, 5% CO_2. After two washes in complete medium, the cells were seeded in microtiter wells in a final volume of 50 μl. Triplicates of the effector-to-target-cell-ratios of 100:1, 50:1, 25:1 and 12.5:1 were incubated for 4 hours. The plates were centrifugated and 100 μl of supernatant were collected and counted in a gamma-counter (LKB 1275 Minigamma). The spontaneous release was determined

from control tubes containing only target cells and medium. Maximum release was obtained by addition of Triton 10%. Specific ^{51}Cr release was calculated applying the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{Exp. rel} - \text{Sp. rel}}{\text{Max rel} - \text{Sp. rel}} \times 100$$

Exp. rel: activity of released ^{51}Cr from target cells in the presence of effector cells.

Sp. rel: activity of ^{51}Cr released spontaneously under identical conditions from target cells alone. Max. rel: maximum activity of ^{51}Cr released when all target cells are destroyed.

In the experiments performed to evaluate the *in vitro* effects of Ni on NK cell activity, PBL were incubated for 2, 4 and 6 hours in presence or in absence of 100 μg/ml of $Ni\frac{I}{N}$ sulfate ($NiSO_4$). Triplicates of the effector to target cells ratios of 50:1, 25:1, 12.5:1 were incubated for four hours at the same conditions. Supernatants were then separated and NK cells activity measured as above described.

Statistical analysis

Results are expressed as the mean of triplicate measurements of NK cell activity. The Student *t* test was used to compare the two independent groups of individuals studied here (Ni-allergic patients and healthy donors matched for sex and age). Differences in the incidence of infections were analysed with the Fisher exact test.

RESULTS

Impaired NK cell activity in Ni sensitized patients

The peripheral distribution of NK cells was evaluated by cytofluorometry staining cells with anti-CD56 and anti-CD3 mAbs. Both percentage and absolute numbers of peripheral NK cells came within the normal range (data not shown). NK cell activity was measured at basal conditions at three effector to target cell ratios (50:1, 25:1, 12.5:1) by a standard ^{51}Cr release assay. Results are shown in Fig. 1. Patients with Ni intolerance showed a baseline NK cell activity significantly lower than controls. This was seen at each effector-to-target cell ratio used with values of cell cytotoxicity approximately 30% lower than observed in controls (more exactly -31.8%, -31.7% and -34.8%, for the effector to target cell ratio 50:1, 25:1 or 12.5:1, respectively). Median NK cell activity was in $Ni\frac{I}{N}$ allergic patients 34.4 (range 6.4-67) whereas in the control group it reached the value of 55.9 (range 13.2-84).

Effect of nickel sulphate on NK cell activity in vitro

In order to ascertain whether an NK cell of Ni-allergic patients was intrinsically more susceptible to the inhibitory effects exerted by $Ni\frac{I}{N}$ sulphate, we performed *in vitro* experiments culturing PBL from both Ni-allergic patients and healthy controls in the presence or absence of $Ni\frac{I}{N}$ sulphate. Data reported in Fig. 2 indicate that NK cells from Ni-allergic patients did not significantly differ from NK cells obtained from apparently healthy donors in terms of susceptibility to Ni effects. In fact, the NK cell cytotoxicity of Ni-allergic patients was reduced *in vitro* by the addition of $Ni\frac{I}{N}$ sulphate of an extent similar to that observed in the corresponding dilution of healthy controls. However, due to the lower basal activity, Ni-allergic patients reached a very low level of NK cell activity following incubation with $Ni\frac{I}{N}$ sulphate.

Increased recurrences of HSV in Ni-allergic patients

Since NK cells play a fundamental role in limiting viral infections before the development of adaptive immune responses, we looked for a positive association between Ni hypersensitivity and susceptibility to viral infections. To this aim, our first approach was a retrospective analysis based on clinical notes of a larger cohort of Ni-allergic patients (n = 158) followed by our Section of Clinical Immunology and Allergy. Although this approach generally leads to an underesteem of the parameter investigated, we

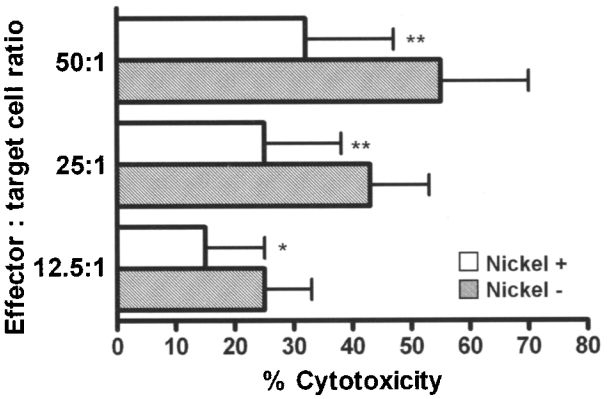

Fig. 1. *NK cell activity at basal condition in nickel intolerant patients (Ni$^+$) and in healthy controls (Ni$^-$). NK cell activity was measured in triplicate. The effectors to target cell ratios are indicated.*$p<0.05$, **$p<0.01$.*

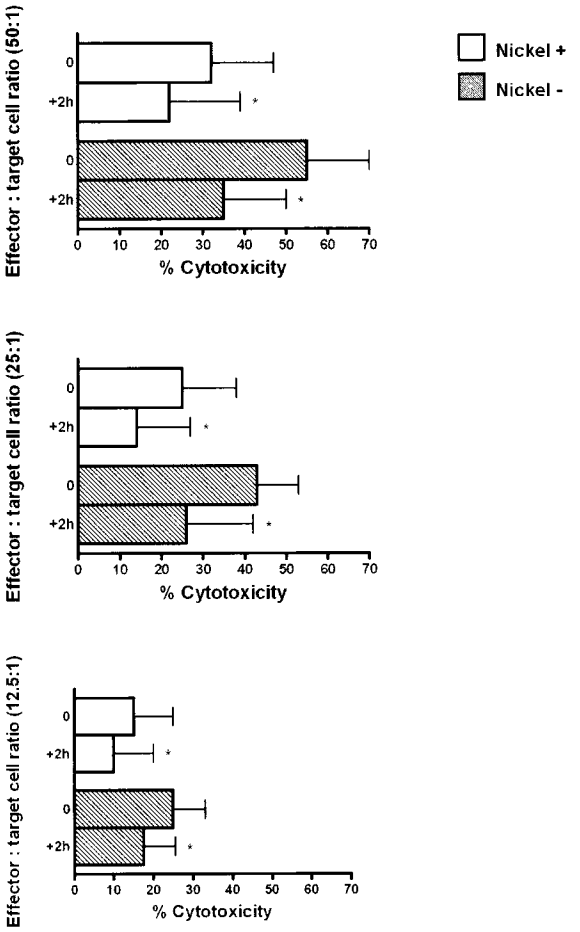

Fig. 2. *NK cell activity of* $Ni\frac{1}{N}$ *intolerant patients (Ni⁺) and healthy donors (Ni⁻) after 2 hours of incubation in presence or in absence of* $Ni\frac{1}{N}$ *sulphate at the concentration of 100 μg/ml. * $p<0.05$.*

found an increased prevalence of HSV recurrences as compared to data recently reported in a very large cohort of young in USA (8.8% in our patients vs 1.4% in the casuistry of Shulman et al.) (10). Interestingly, we also found, besides the increased number of HSV labialis recurrences, an association between ACD and vaginitis, particularly those sustained by *Candida albicans*. Therefore, we administered to the patient population enrolled in this study a questionnaire specifically designed to unveil the real prevalence of infectious disease in Ni-allergic patients. The results are reported in Table I showing a prevalence of Herpes labialis recurrences in 15% of ACD patients. Serum IgG, IgA and IgM levels were within normal range (data not shown).

DISCUSSION

A large number of human NK cell deficiency states exist, providing insight into the key role exerted by NK cells in containing infections diseases and cancers (11-12). Similarly to other immunodeficiency diseases, NK cell deficiencies can be due to both quantitative and qualitative defects. In this work we focused our attention to the immunological correlates of Ni hypersensitivity. In particular we investigated the NK cell activity and the susceptibility to infectious diseases of Ni-allergic patients. Increasing evidences indicate Ni as a metal ione capable to deeply affect the functionality of the immune system (5,13). In addition to possess a very high immunogenic potential, Ni can exert an immunosuppressive effect by acting at multiple cell levels. Among the cells belonging to the immune system primarily affected by Ni there are T and NK lymphocytes. Regarding the NK cells, it has been demonstrated, before in the mouse model and after in humans, that Ni can suppress the NK cytotoxicity (3,14-15). These data on NK cell activity are in agreement with those reported by Daniels et al. (4) indicating an immunosuppressive activity of Ni on NK cells resulting in increased mortality of mice inoculated with sublethal doses of MCMV. Similary Ilback et al. (16) showed a direct contribution of Ni to the progression of target organ pathology in coxsackievirus B3 (CB3) infection-induced diseases such as diabetes and myocarditis in Balb/c mice. Since a role of NK cells in anti-viral immunity has been demonstrated most convincingly in protecting against cytomegalovirus and other herpesviruses (8), it has been our interest to explore the possibility that $Ni\frac{1}{N}$ intolerant patients could have an increased susceptibility of HSV recurrences. In fact, we recently described an impaired NK activity in $Ni\frac{1}{N}$ allergic patients theoretically consistent with a decreased anti-viral immunity. In the present work we confirm and extend our data on depressed NK activity in $Ni\frac{1}{N}$ allergic patients and suggest for the first time a link between Ni hypersensitivity and HSV recurrences. The prevalence we report here of HSV recurrences in patients with Ni-related ACD is significantly higher than observed in the general population.

Patients	Number of patients	Prevalence of HSV recurrences
All $Ni^{\frac{I}{N}}$ allergic patients followed by our centre (data obtained from clinical notes)	158	8,8%
$Ni^{\frac{I}{N}}$ allergic patients enrolled in the present study (data obtained from a specific questionnaire)	55	15%
Healthy controls	25	1%

Table I. *Prevalence of HSV-1 recurrences in* $Ni^{\frac{I}{N}}$ *allergic patients.*
**Clinical notes of a larger cohort of* $Ni^{\frac{I}{N}}$ *allergic patients were firstly examined in order to find any possible linking between Ni intolerance and infectious diseases. Only the most relevant infection disease associated with Ni allergy is shown.*
***A questionnaire based on anamnestic data obtained from clinical notes was administered to the patient population enrolled in the present study.*
Recurrences were defined as 6 or more episodes of herpes labialis per year.

Moreover, data obtained from the administration of a questionnaire specifically designed to demonstrate an association between Ni allergy and susceptibility to infectious diseases pointed out a causative role for Ni intolerance in the pathogenesis of recurrent *Candida albicans* vaginitis. This possibility, consistent with previous reports indicating that, among PBL, NK cells have the major natural anti-candidial activity(17), this is currently under investigation. Finally, our data allow to hypothesize alternative therapeutic strategies in patients with Ni allergy and in patients with HSV recurrences or vaginitis by *Candida albicans*. In particular we refer to the possibility, in addition to avoid the intake of Ni, of favouring or supplementing the dietary intake of Zinc and Manganese, two elements capable of potentiating NK cell activity and to counteract the immunosuppressive effects of Ni (18-23).

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